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(54) Title: CHIMERIC TOXINS BINDING TO THE GnRH RECEPTOR

(57) Abstract

Analogs of GnRH are functionalized with unique linking groups so that they may be coupled to a cell-killing molecule. The chimeric toxin comprises a GnRH analog, a linking group and a toxin component which are administered to male and female animals where it is transported to organs containing cells with GnRH receptors such as pituitary glands in order to reduce secretions of sex steroids which results in sterility or in the reduction of tumors that require sex steroids for growth. The instant compounds are used as sterilizing agents and anticancer agents. The GnRH derivatives modified with the instant linking groups provides an advantage over prior chimera prepared by conjugation in that upon amino acid analysis of the conjugate, the modified GnRH derivative releases an unnatural amino acid which is readily quantified thus revealing the degree of conjugation between the GnRH analog and the toxin.

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TITLE OF THE INVENTION

CHIMERIC TOXINS BINDING TO THE GnRH RECEPTOR

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of our copending application Serial No. 07/836031 filed 14 February 1992.

BACKGROUND OF THE INVENTION

Considerable interest exists with respect to the subject of sterilization of animals. This is especially true of those concerned with veterinary medicine and animal husbandry, particularly as they relate to the subject of sterilization of domestic animals such as dogs, cats, cattle, sheep, horses, pigs, and the like. Various methods have been developed over the years to accomplish sterilization. For example, with respect to male cattle, the most widely used procedure for eliminating problems of sexual or aggressive behavior is sterilization through surgical castration. This is done in various ways, e.g., crushing the spermatic

cord, retaining the testes in the inguinal ring, or use of a rubber band, placed around the neck of the scrotum, to cause sloughing off of the scrotum and testes. However, most of these "mechanical" castration methods have proven to be undesirable in one respect or another; for example they (1) are traumatic, (2) introduce the danger of anesthesia, (3) are apt to produce infection, and (4) require trained personnel. Moreover, all such mechanical castration methods result in complete abolition of the testes and this of course implies complete removal of the anabolic effects of any steroids which are produced by the testes and which act as stimuli to growth and protein deposition.

These drawbacks have caused consideration of various alternative sterilization techniques such as the use of chemical sterilization agents. However, the use of chemical sterilization agents has its own set of advantages and disadvantages. On the positive side, chemical sterilization eliminates the stress and danger associated with mechanical castration. Chemical sterilization also has the added advantage of allowing for retention of certain anabolic effects resulting from a continued presence of low levels of circulating testosterone. This is especially valuable in the case of animals raised for human consumption since circulating testosterone promotes growth, efficiency of feed conversion and protein deposition. Unfortunately, there are several disadvantages associated with chemical sterilization. For example chemical sterilization is often temporary rather than permanent; it also

sometimes produces extremely severe, and even fatal, side effects.

In WO 9009799 to Nett certain GnRH analogs are coupled to a variety of toxins through an optional linking group consisting of 2-iminothiolane, SPDP (N-succinimidyl-3-(2-pyridyldithio/propionate), bis-diazabenzidine and glutaraldehyde. The compounds are disclosed as sterilizing agents.

In addition, Myers et al Biochemical Journal 227:1 pg 343 (1985) discloses a conjugate of a GnRH analog and the diphtheria A chain and pokeweed antiviral toxins coupled through SPDP.

Since toxins commonly have multiple ligand attachment sites, (generally amino or carboxy groups) and the linkage chemistry is not selective with regard to the number of GnRH molecules activated, there is no chemical control of the degree of conjugation. That is, conjugation can occur to one or through multiple attachments. By conventional chemistry, determination of the number of attachments of ligands is difficult or impossible. An unverified assumption of these prior works is that all toxin conjugates, regardless of their stiochiometry, are efficacious or equally efficacious.

Thus, while it is known generally that a toxin can be coupled to a GnRH analog through a linking group, the instant linking groups are unique and have been found to offer unique advantages in the efficacy of the final toxin conjugate, in the process for the manufacture thereof and in the analysis of conjugated peptide and toxin.

In addition, various constructs of bacterial or plant toxins have been prepared in attempts to specifically target the toxin to certain cells or organs.

U.S. patent 4,545,985 teaches that Pseudomonas exotoxin A can be chemically conjugated to an antibody or to epidermal growth factor. While this patent further teaches that these conjugates can be used to kill human tumor cells, these chemically linked toxins have been shown to have undesirable, nonspecific levels of activity.

U.S. patent 5,036,047 teaches that free LHRH (alternate nomenclature for GnRH) can be administered along with an immunogenic conjugate of LHRH and a specific nonapeptide or decapeptide. The administration is reported to induce reversible sterilization.

U.S. patent 4,664,911 teaches that antibodies can be conjugated to the A chain or the B chain of ricin which is a toxin obtained from plants. Patent 4,664,911 further teaches that these conjugates can be used to kill human tumor cells.

U.S. patent 4,675,382 teaches that hormones such as melanocyte stimulating hormone (MSH) can be linked to a portion of the diphtheria toxin protein via peptide bonds. Patent 4,675,382 further teaches that the genes which encode these proteins can be joined together to direct the synthesis of a hybrid

fusion protein using recombinant DNA techniques. This fusion protein has the ability to bind to cells that possess MSH receptors.

Murphy, et al., PNAS USA 83:8258-8262 (1986). This article teaches that a hybrid fusion protein produced in bacteria using recombinant DNA technology and consisting of a portion of the diphtheria toxin protein joined to alpha-melanocyte-stimulating hormone will bind to and kill human melanoma cells.

Allured, et al., PNAS USA 83:1320-1324 (1986). This article teaches the three dimensional structure of the Pseudomonas exotoxin A protein.

Hwang, et al., Cell 48:129-136 (1987). This article teaches that the Pseudomonas exotoxin A protein can be divided into three distinct functional domains responsible for: binding to mammalian cells, translocating the toxin protein across lysosomal membranes, and ADP ribosylating elongation factor 2 inside mammalian cells. This article further teaches that these functional domains correspond to distinct regions of the Pseudomonas exotoxin A protein.

Chaudhary, et al., PNAS USA 84:4538-4542 (1987). This article teaches that hybrid fusion proteins formed between PE-40 and transforming growth factor-alpha and produced in bacteria using recombinant DNA techniques will bind to and kill human tumor cells possessing epidermal growth factor receptors.

European patent application 0 261 671, published 30 March 1988, teaches that a portion of the Pseudomonas exotoxin A protein can be produced which lacks the cellular binding function of the whole Pseudomonas exotoxin A protein but possesses the translocating and ADP ribosylating functions of the whole Pseudomonas exotoxin A protein. The portion of the Pseudomonas exotoxin A protein that retains the translocating and ADP ribosylating functions of the whole Pseudomonas exotoxin A protein is called Pseudomonas exotoxin - 40 or PE-40. PE-40 consists of amino acid residues 252-613 of the whole Pseudomonas exotoxin A protein as defined in Gray, et al., PNAS USA 81:2645-2649 1984. This patent application further teaches that PE-40 can be linked to transforming growth factor-alpha to form a hybrid fusion protein produced in bacteria using recombinant DNA techniques.

Kelley, et al., PNAS USA 85:3980-3984 (1988). This article teaches that a hybrid fusion protein produced in bacteria using recombinant DNA technology and consisting of a portion of the diphtheria toxin protein joined to interleukin 2 functions in mice to suppress cell mediated immunity.

Bailon, Biotechnology, pp. 1326-1329 Nov. (1988). This article teaches that hybrid fusion proteins formed between PE-40 and interleukin 2 and produced in bacteria using recombinant DNA techniques will bind to and kill human cell lines possessing interleukin 2 receptors.

Edwards, et al., Mol. Cell. Biol. 9: 2860-2867 (1989) describe the preparation of the modified TGF-alpha - PE_{40} hybrid molecules that have been found to have utility in treating bladder tumor cells.

Heimbrook, et al., Proc. Natl. Acad. Sci. USA 87: 4697-4701 (1990) describe the in vivo efficacy of modified TGF-alpha - PE_{40} in significantly prolonging the survival of mice containing human tumor cell xenografts.

A term for the selective delivery of chemotherapeutic agents to specific cell populations is "targeting". Drug targeting to specific cells can be accomplished in several ways. One method relies on the presence of specific receptor molecules found on the surface of cells. Other molecules, referred to as "targeting agents", can recognize and bind to these cell surface receptors. These "targeting agents" include, e.g., antibodies, growth factors, or hormones. "Targeting agents" which recognize and bind to specific cell surface receptors are said to target the cells which possess those receptors. For example, pituitary cells that release lutenizing hormone (LH) possess a protein on their surfaces that recognizes and binds with GnRH. GnRH is therefore, a "targeting agent" for these cells.

"Targeting agents" by themselves do not kill cells. Other molecules including cellular poisons or toxins can be linked to "targeting agents" to create hybrid molecules that possess both cell targeting and cellular toxin domains. These hybrid

molecules function as cell selective poisons by virtue of their abilities to target selective cells and then kill those cells via their toxin component. Some of the most potent cellular poisons used in constructing these hybrid molecules are bacterial toxins that inhibit protein synthesis in mammalian cells. Pseudomonas exotoxin A is one of these bacterial toxins, and has been used to construct hybrid "targeting - toxin" molecules (U.S. Patent 4,545,985).

Pseudomonas exotoxin A intoxicates mammalian cells by first binding to the cell's surface, then entering the cell cytoplasm and inactivating elongation factor 2 which is a cellular protein required for protein synthesis. Pseudomonas exotoxin A has been used to construct anticancer hybrid molecules using monoclonal antibodies and protein hormones. However, one problem with these hybrid molecules is that they exhibit toxicity towards normal cells. At least part of the toxicity associated with hybrid molecules containing Pseudomonas exotoxin A is due to the ability of Pseudomonas exotoxin A by itself to bind to and enter many types of mammalian cells. Therefore, hybrid molecules formed between Pseudomonas exotoxin A and specific "targeting agents" can bind to many normal cells in addition to the cells recognized by the "targeting agent". One method of dealing with this problem is to modify Pseudomonas exotoxin A so that it is no longer capable of binding to normal cells. This can be accomplished by removing that portion of the Pseudomonas exotoxin A molecule

which is responsible for its cellular binding activity. A truncated form of the Pseudomonas exotoxin A molecule has been prepared which retains the ability to inactivate elongation factor 2 but no longer is capable of binding to mammalian cells. This modified Pseudomonas exotoxin A molecule is called Pseudomonas exotoxin - 40 or PE_{40} (Hwang, et al., Cell 48:129-136 1987).

The instant invention utilizes various constructs of GnRH, a linking group and a toxin. However, unlike the prior art constucts which rely upon the techniques of biotechnology and recombinant DNA, the instant invention describes the preparation of site-specific toxin constructs using the techniques of synthetic organic chemistry.

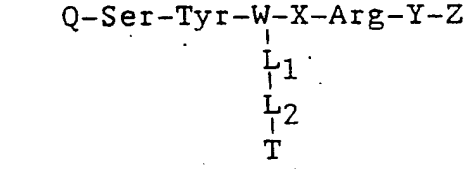
In addition, the prior art constructs were difficult to analyze for amino acid content because there was no way of knowing how many of the GnRH peptides bonded with the toxin since the toxins, being polypeptides; generally had more than one site with which the GnRH, or othe peptide, could bond. The instant linking groups, upon degradation, release a specific marker in the form of an unnatural amino acid. The amount of this marker allows the facile calculation of the exact ratio of peptide groups to toxins.

SUMMARY OF THE INVENTION

This invention is concerned with an agent which is selectively toxic to the LH releasing cells of the pituitary. This selective toxicity is achieved by first removing, from a general toxin, the domain involved in binding to cells which results in greatly reduced general toxicity. This binding domain is replaced, in our invention, with a group (GnRH) which specifically binds to LH releasing cells and thus creates a very specific, new binding domain. The conjugation of these two moieties is accomplished with specific and improved linking groups that also aid in the analysis of the peptide to toxin ratio. This new, chimeric conjugate is now selectively toxic to those cells in the pituitary gland which release luteinizing hormone (LH). The death of these cells and the concomitant inability to generate LH results in the irreversible sterilization of the animal. While a broad category of toxic agents can be utilized in the chimeric toxin conjugate, conjugates based on Pseudomonas exotoxin A are preferred.

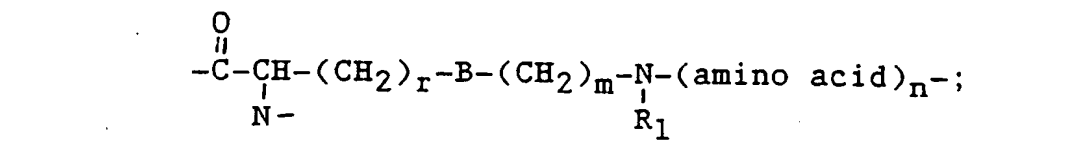
DESCRIPTION OF THE INVENTION

The chimeric toxic agents are best described in the following structural representation:



where Q is PyroGlu-His-Trp, N-acetyl-4-Cl-Phe^{1,2}-Trp or 3-indolylpropionyl;

W is the D or L amino acid with a pendant linking functionality such as



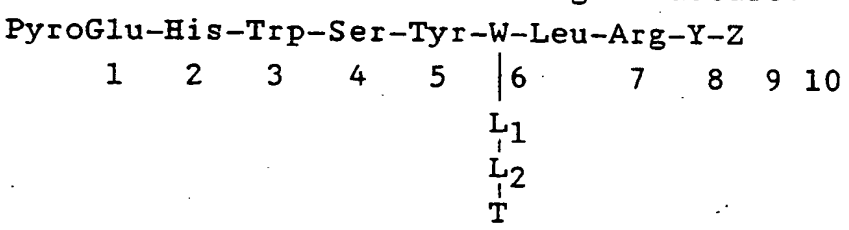
where

r is 1 or 2;
m is 1 to 4;
n is 0 or 1;
B is CH_2, O, S or N; and
R_1 is hydrogen, C_1-C_6alkyl or C_3-C_8cycloalkyl;

X is Leu or Nle;
Y is Pro or 4-hydroxy-Pro; and
Z is Gly, NH_2, D-Ala-NH_2, NH-Et, NH-Pr or Aza-Gly-NH_2.

L_1, L_2 and T are as defined below.

Preferred compounds of the instant invention are realized in the following structure:



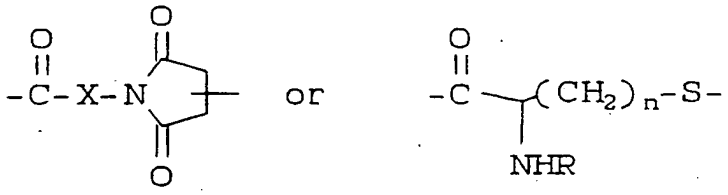
where the decapeptide is GnRH with the normal 6-position amino acid (W=Gly) deleted and replaced by W=D-Lys or D-Orn. The use of D-Lys^6-GnRH is a preferred targeting agent with which to bind the toxic construct to the LH releasing pituitary cells, it will be recognized that variations of D-Lys^6-GnRH

that will still bind to the GnRH receptor of the pituitary will be useful in this invention. All that is required is that the 6-position amino acid possess an amino group for binding to the linking group and that the remainder of the peptide bind to the GnRH receptor on the pituitary gland cells.

Z in the above formula is Gly-NH_2, ethylamide, or Arg-Gly NH_2;

Y is Pro or 4-hydroxy-Pro;

L_1 and L_2 are independently



where X is C_1-C_5 alkylene, phenyl or C_5-C_6 cycloalkylene;

R is C_1-C_3 alkanoyl;

n is 1 or 2; and

T is a toxin group; provided that the carbonyl ends of each of L_1 and L_2 are bonded to either the GnRH derivative or to the toxin.

It will be appreciated by those skilled in the art that 4-hydroxy-Pro can exist as D and L isomers and as cis and trans isomers. All such isomers, and the racemic mixture of the D and L isomers are intended to be included in this invention.

The toxin can be any toxin that is capable of destroying the LH releasing cells of the pituitary, referred to as gonadotrophs. The toxins can be plant derived toxins, bacteria derived toxins or chemical toxins. Examples of plant derived toxins are ricin, modeccin, abrin, pokeweed antiviral protein, α-amanatin, gelonin ribosome inhibiting protein, (RIP) or RIP derived from wheat, corn, rye, flax and the like. Examples of bacteria derived toxins are diphtheria toxin, Pseudomonas exotoxin, shiga toxin and the like. Examples of chemical toxins are melphalan, methotrexate, nitrogen mustard, doxorubicin, daunomycin and the like.

The preferred toxins are those derived from Pseudomonas exotoxin. The most preferred toxins are those segments of Pseudomonas exotoxin wherein the binding domain has been deleted or partially deleted so that the toxin retains its potential for cell toxicity but that the toxin lacks the ability to bind to animal cells, except when coupled with the GnRH targeting agent.

One example of such a Pseudomonas toxin has had amino acids 1-252 deleted, which comprises most or all of the binding region and retaining amino acids 253-613 which contain the cell translocation region and the toxin region. This Pseudomonas exotoxin fragment has been identified as PE-40 - See Hwang et al., supra, Kondo et al J. Biol Chem 263 pg 9470-9475 (1988), Chaudharg et al, DNAS-USA, 87 pg 308-312 (1990) and US Patent 4892827 to Pastan et al.

The Pseudomonas exotoxin fragment PE-40 has been further modified by removing additional amino acids 365-380 and further providing the point mutations of deleting one or more of the Lys amino acids and replacing these with a different amino acid that will not bond with L_1 or L_2. Since, however, Lys is the amino acid that is bonded to the L_1 or L_2 at least one Lys is preferred to be retained in the Pseudomonas exotoxin peptide fragments. One such modified PE-40 has been designated PE-38M and it is shown in Table 1 where the numbers above the peptide sequence refer to the Pseudomonas exotoxin sequence.

The various Pseudomonas exotoxin fragments are prepared using the techniques of biotechnology and recombinant DNA. However, once the Pseudomonas exotoxin has been prepared, it is bonded to the linking groups L_1 and L_2 and the D-Lys^6-GnRH using synthetic organic chemical techniques.

Sequence Listing Table PE-38M

253 300
MLQGTKLMAEEGGSLAALTAHQACHLPLETFTRHRQPRGWEQLEQCGYPVQRLVALYLAAR
350 364
LSWNQVDQVIRNALASPGSGGDLGEAIREQPEQARLALTLAAAESERFVRQGTGNDEAGAAN
381 400
GPADSGDALLERNYPTGAEFLGDGGDVSFSTRGTQNWTVERLLQAHRQLEERGYVFVGYHGTFL
450 500
EAAQSIVFGGVRARSQDLDAIWRGFYIAGDPALAYGYAQDQEPDARGRIRNGALLRVYVPRSSL
550
PGFYRTSLTLAAPEAAGEVERLIGHPLPLRLDAITGPEEEGGRLETILGWPLAERTVVIPSAIP
600 613
TDPRNVGGDLDPSSIPDQEQAISALPDYASQPGQPPREDLR

Additional toxins are _____ from the Pseudomonas exotoxins by the selection ____ of the amino sequence such as ....

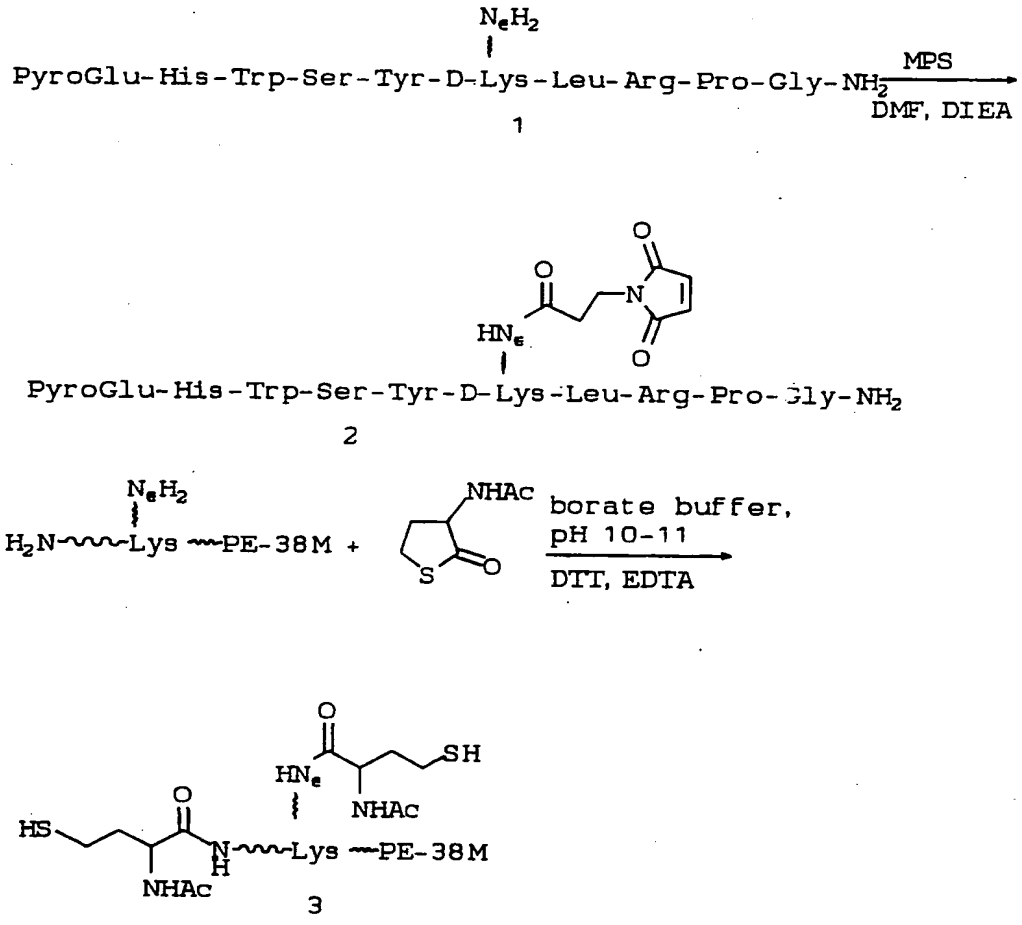
DESCRIPTION OF THE DRAWINGS

Figure 1 is a mass spectrometric analysis of the toxin conjugate of DLs^{6}-GnRH coupled to PE38 with from 1 to 5 GnRH moieties per toxin. The six membered peaks show an increasing molecular weight order, the unconjugated protein and the protein conjugated sequentially with from 1 to 5 GnRH analogs. The analysis was carried out using Laser Time-of-flight (TOF) technique or a matrix consisted matrix description TOF mass spectrometer.

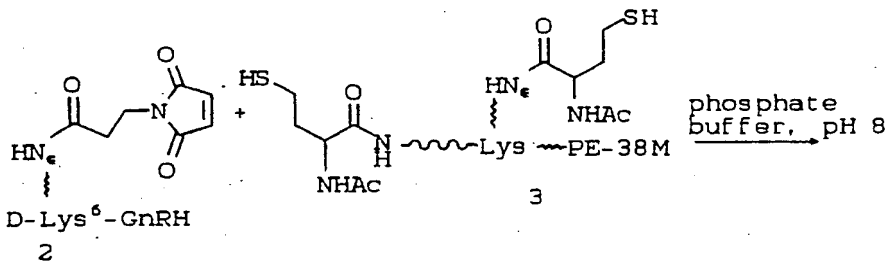
Figure 2 is a mass specromative analysis of the PE38 starting material of approximately 38K molecular weight. The two smaller peaks represent doubly or tripled charged molecules.

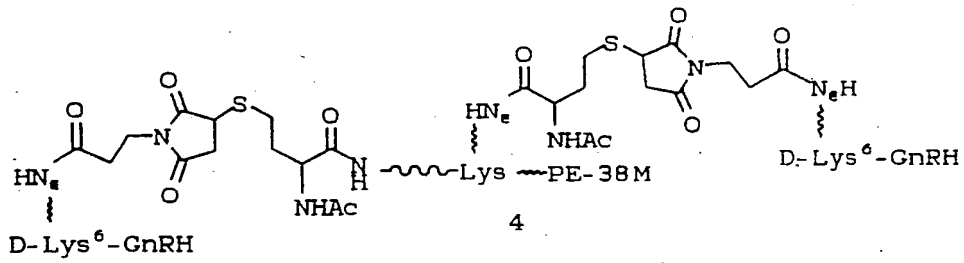
Figures 3, 4 and 5 are SDS-PAGE Gel electrophoreis of the conjugates of PE38 D-Lys^6 GnRH (Figures 3 and 4) or PE38M and D-Lys^6-GnRH (Figure 5). The preparation of the instant toxin conjugates is shown in Reaction Scheme 1 using D-Lys^6 GnRH and PE-38M for exemplification.

REACTION SCHEME 1



REACTION SCHEME I (CONT'D)





In this reaction sequence the GnRH is first modified with one of the L_1 and L_2 linking groups and the modified Pseudomonas exotoxin peptide is modified with the other of the L_1 and L_2 linking groups. The L_1 and L_2 linking groups are attached to all free or unprotected primary amines on the GnRH derivative and the Pseudomonas exotoxin. In the case of the D-Lys^{6}GnRH the linking group is bonded to the amine at the end of the alkyl chain of the Lys. Since the N-terminus of the D-Lys^{6}GnRH is a pyroglutamyl, no reaction can occur since no free primary amine is present. In the case of the Pseudomonas exotoxin, reaction can occur at any Lys present. The PE-38M modified protein has only one Lys but other modified Pseudomonas exotoxins may have more than one. In addition, the N-terminus of the Pseudomonas exotoxin is a free amine which is available for reaction with L_1 and L_2. If no other Lys were present on the modified Pseudomonas exotoxin, coupling with the D-Lys^6-GnRH would still occur since the N-terminus amine would still provide the linking site. With more than one amine, multiple couplings of the D-Lys^6-GnRH will occur.

In Reaction Scheme 1 the first step is the reaction of D-Lys^6-GnRH with L_1 which for purposes of illustration is shown as the maleimidoyl alkanoyl group. The other linking group, N-alkanoyl cysteine or N-alkanoyl homocysteine could also be used. The D-Lys^6-GnRH is prepared using known peptide synthesis techniques, preferably the solid phase peptide synthesis.

The reaction for the preparation of the L_1-D-Lys^6-GnRH is carried out using an active ester of the maleimidoyl alkanoyl group. Preferred esters are the esters made from maleimidoyl alkanoic acid and N-hydroxy succinimide, pentafluorophenol or p-nitro phenol. The ester with N-hydroxy succinimide is most preferred. The reaction is carried out in a polar solvent with a base selected from either (a) a non-nucleophilic organic base such as N,N-diisopropyl ethylamine or (b) a weak inorganic base such as sodium or potassium carbonate. The polar solvent can be N,N-dimethylformamide, water, acetonitrile or mixture thereof. N,N-Dimethylformamide is preferred. The reaction is carried out at from 0 to 25°C, preferably at room temperature and is generally complete in from 10 to 90 minutes. The work-up of the reaction is to initially neutralize the base present with an acid such as trifluoroacetic acid, and the pH of the mixture is brought to about 2-4. The product is than isolated using techniques known to those skilled in the art.

In the next step of the reaction scheme, the Pseudomonas exotoxin is reacted with the other linking group L_2 at any and all of the unprotected primary amines. For purposes of illustration the Pseudomonas exotoxin is shown as PE-38M which has two primary amines available for reaction, the N-terminus amine and the epsilon amine or the Lys, shown as N_ε. With such an arrangment two equivalents of the L_1-D-Lys^{6}GnRH will react with each equivalent of the PE-38M.

The reaction is carried out in an aqueous buffer which provides for a pH of greater than 10. Borate buffer solution with a pH of about 11 is preferred. Included in the reaction mixture preferably is dithiothreitol and/or the disodium salt of ethylenediaminetetraacetic acid. These reagents are generally added in considerable excess in order to prevent the reactive mercapto group from forming a disulfide bond with like groups. For the same reason, the reaction is carried with the strict exclusion of oxygen, generally by using a nitrogen atmosphere. The reaction is carried out at from 0 to 25°C, preferably room temperature, and is generally complete in from 5 to 18 hours. Before the L_2-Pseudomonas exotoxin can be conjugated with the L_1-D-Lys^6-GnRH, the dithiothrietol, the ethylene-diaminetetraacetic acid disodium salt and any hydrolyzed N-alkanoyl homocysteine thiolactone must be removed. Thus the reaction mixture is purified of such reagents prior to the next step. The most convenient method for doing so is to dialyze the reaction mixture. The dialysis solution free of extraneous reagents is used in the next step without further treatment.

The final step is the coupling of the L_1 D-Lys^{6}GnRH with the L_2-Pseudomonas exotoxin which is carried out under nitrogen at a pH of from 8 to 10 with a excess of the L_1 D-Lys^{6}GnRH. Generally from 2 to 20 equivalents of the GnRH reagent are used for each equivalent of the L_1 substituent on the Pseudomonas exotoxin. The reaction is generally very fast and is complete in just 1-5 minutes although further aging of up to 2 hours has not been found to

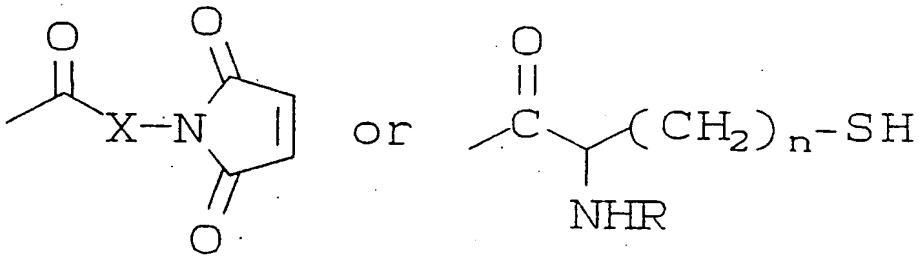
be detrimental. The coupled D-Lys^{6}GnRH L_1-L_2-pseudomonas exotoxin is isolated using techniques known to those skilled in the art. It has been found that dialysis of the reaction mixture is a convenient method for the removal of unwanted products. Since the conjugated product will generally be administered by injection, the resultant dialysis solution may be sterile filtered and used directly for percutaneous administration.

The intermediate compounds 2 and 3 are important aspects of the instant invention and represent novel compounds. The novel intermediates are realized in the following structural formulae:

p-Glu-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Y-Z
L

and L-T

where L is independently

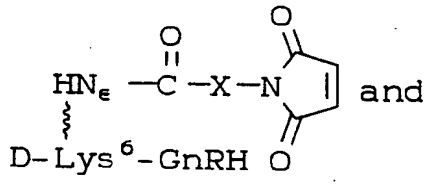


where

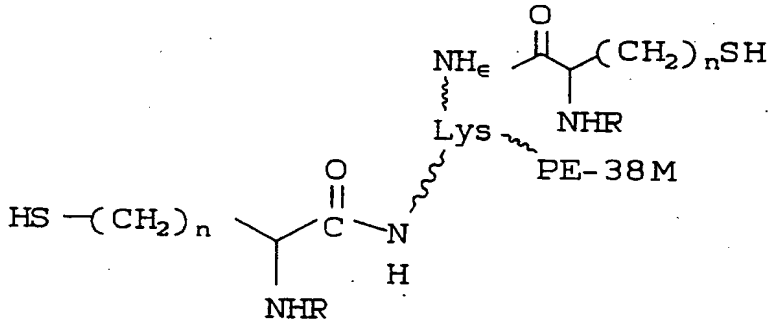
X is C_1-C_5alkylene, phenyl or C_5-C_6cycloalkylene;
R is C_1-C_3alkanoyl;
n is 1 or 2; and
T is a toxin.

The linking groups, L, are bonded to the available primary amines of the D-Lys^6-GnRH and the toxin, preferably Pseudomonas exotoxin A or fragment thereof. Where, as in the Pseudomonas exotoxin, more than one primary amine is available, more than one L group will be reacted therewith.

While either value of L may be bound to either the D-Lys^6-GnRH or the toxin, it is preferred to bond the maleimido alkanoyl group to the GnRH and the N-alkanoyl homocysteinyl group to the toxin. The most preferred toxin is PE-38M and the most preferred GnRH derivative is D-Lys^6-GnRH, thus, the most preferred intermediates of the instant invention are:



and



The instant site-specific toxins which connect a GnRH analog with a toxin through a unique linking group offer significant advantages in the preparation and analysis of the toxin conjugates. The toxin moieties often have more than one amine function which can be coupled to the GnRH analog through various linking groups (See Nett et al). However, in preparing such toxin conjugates, it is not certain if all of the amine functions, generally found on the amino acid lysine, will react with the GnRH linking group, thus at the completion of the reaction the conjugate will have to be analyzed to determine the number of GnRH moieties that have been bonded to the toxin. A further complication arises when, as often occurs, the resultant conjugate is actually a mixture of conjugates with differing numbers of GnRH analogs coupled to the toxin. The analysis of the toxin conjugates is usually done through amino acid analysis, however, since the GnRH and toxin all break down to normal amino acids, the determination of the number of GnRH moieties bonded to the toxin is a very long and tedious process.

The instant linking group, however, under amino acid analysis breaks down to beta-alanine, an unnatural amino acid. Thus during amino acid analysis, the ratio of lysine to beta-alanine is determined which reveals the extent of the conjugation. This is a much more direct and accurate method of determining the degree of conjugation which greatly facilitates the use of the toxin conjugates as chemical sterilization agents.

Applicants have found that the GnRH conjugates of the instant invention are particularly effective in causing the toxic compound T to be specifically targeted to the gonadotropin-secreting cells of the anterior pituitary gland. Indeed, they are the only cells to which the gonadotropin-releasing hormone portion of the conjugate will bind. Hence, these toxic compounds, bound to an analog of gonadotropin-releasing hormone, can be employed to permanently destroy a subpopulation of the anterior pituitary cells and thereby eliminate the gland's ability to secrete gonadotropins. This in turn causes the animal's gonads to atrophy and lose their ability to function for reproductive purposes. That is to say that, without functioning gonadotrophs, an animal is not able to secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH) and thus is rendered sterile. Applicants have postulated that the compounds of this patent disclosure inhibit synthesis of LH, and presumably other proteins made by gonadotrophs, because they tend to inhibit all protein synthesis once these compounds gain entry into a cell. It should also again be noted that applicants' compounds allow "chemical castration" to be employed in place of surgical castration.

Thus, the use of these compounds has great utility in human medicine as well as in veterinary medicine. This follows from the fact that there are several important biological reasons for employing castration and antifertility drugs in humans. For example, breast and prostate cancers are but two

examples of sex steroid-dependent tumors which respond to such hormonal manipulation. At present, the only reliable way to inhibit steroid-dependent tumor growth is through administration of counter-regulatory hormones (e.g., DES in prostate cancer), sex-steroid hormone binding inhibitors (e.g., tamoxifen in breast cancer) or surgical castration. Thus the potential medical uses of such chemical castration compounds are vast and varied. For example, prostate cancer remains an important cause of cancer deaths and represents the second leading cancer of males. The present palliative treatment for advanced prostate cancer cases involves reduction of serum testosterone/DHT levels through use of surgical castration. It should also be noted that for purposes of disease and/or fertility control, especially in humans, it may be desirable to use applicant's compounds to ablate pituitary gonadotrophs in conjunction with other modes of treatment. For example, it is anticipated that chronic administration of progestins and estrogens to females and androgens to males might be necessary to prevent loss of secondary sex characteristics, behavior and osteoporosis. However, through judicious use of the herein disclosed compounds, especially in combination with appropriately administered sex steroids, desirable antifertility effects can be achieved. Another area of application in human medicine is treatment of endometriosis. This condition, which produces painful growth of endometrial tissue in the female peritoneum and

pelvis also responds to inhibition of sex steroid synthesis. Those skilled in this art will also appreciate that the herein disclosed compounds could be used to partially reduce sex-steroid secretions, and thus reduce or eliminate certain hormone related behavior problems while retaining improved growth stimulation.

The dose/time adjustments associated with the use of these compounds can vary considerably; however, these compounds are preferably administered by injection into a mammal in concentrations of from about 0.1 to about 10 milligrams per kilogram of the mammal's body weight. Sterilization may be accomplished with as few as one injection; but multiple treatments (e.g., based upon concentrations of from about 0.03 milligrams once every 4 days to about 1 milligram per kilogram of body weight for 20 days) are alternative sterilization schemes. Furthermore, as sterilization agents, the compounds of this patent disclosure can be used before or after puberty. They too are especially useful in those areas of animal husbandry where the anabolic benefits of non-surgical sterilization techniques can contribute to meat production and/or quality. In one preferred embodiment of this invention the compounds of this invention are administered to male cattle between the ages of about 8 weeks and 20 weeks at least once and in a concentration of from about 0.1 to about 10 milligrams per kilogram of the animal's body weight.

The toxic moieties T of the herein disclosed compounds are obtainable from both natural and synthetic sources. For example, pokeweed antiviral protein can be isolated from leaves of pokeweed plants and purified by gel filtration chromatography. It can then be, by way of example, conjugated to D-Lys^{6}-desGly^{10}-Pro^{9}GnRH-ethylamide via the amino group on the lysine and through a sulfhydryl group introduced into the pokeweed antiviral protein by the instant novel linking groups. In any event, one of the chief advantages of these compounds is their ability to produce permanent sterilization without strong toxic side effects. Hence these compounds may be used on mammals such as human beings, domestic animals, pets or wild animals. Moreover, they can be administered as a single injection which can induce permanent and irreversible sterility in both male and female mammals. However, an alternative approach to achieve sterilization is through multiple injections at lower dosages than those employed in a single treatment or by slow release implants (i.e., biodegradable formulations).

EXAMPLE 1

Procedure for the Preparation of a 6-D-Lys-GnRH/Lys-PE38M Congugate

General: All reagents were used as received by the supplier. In the case of solvents, HPLC-grade was used where available. HPLC (binary gradient) was performed on a Waters 600E system with Waters 484 tunable U.V. detector (Aufs=0.1 analytical or 2.0 preparative scale) and recorded on a Waters 746 Data Module. A Waters WISP™712 autosampler (2000 µL sample loop) was used for analytical samples. A Rheodyne 7125 manual injection port (5000 µL sample loop) was used for preparative samples. A = H_2O, 0.1% TFA; B=CH_3CN, 0.1% TFA. Mass spectra were taken on a Finnegan MAT 90, spectrophotometer (positive ion, NBA matrix).

Abbreviations: Standard amino acid abbreviations are used. RT, room temperature; DCC, 1,3-dicyclohexylcarbodiimide; HOBT, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid; DIEA, N,N-diisopropylethylamine; MPS, ß-maleimidopropionic acid N-hydroxysuccinimide ester; GnRH, gonadotropin releasing hormone; PBS, phosphate buffered saline; DTT, dithiothreitol; EDTA-2Na, ethylenediaminetetraacetic acid disodium salt; NBA, 3-nitro-benzyl alcohol.

6-D-Lys-GnRH,1:PyroGlu-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2:

The peptide was synthesized on Rink amide MBHA resin (0.25 mmol, Amino Tech) by solid phase peptide synthesis (SPPS) using an ABI model 431A

synthesizer and single couplings (DCC/HOBT). The peptide was cleaved (2 h, RT) from the resin using reagent R (1 mL/100 mg resin, TFA/thioanisole/ethanedithiol/anisole, 90:5:3:2). The peptide was precipitated from the concentrated cleavage mixture with diethyl ether and purified by preparative reverse phase HPLC (Waters PrepPak®25 x 10™ C_{18}; 10 mL/min; 10-20% B, 0-20 min.; then 20-35% B, 20-40 min.; λ=230 nm).

6-D-Lys-GnRH: FAB-MS (positive ion, NBA matrix) Calc. M+1 1254.44; Found M+1 = 1254.4

(N_ε-maleimidopropanoyl)-6-D-Lys-GnRH,2:PyroGlu-His-Trp-Ser-Tyr-(N_ε-maleimidopropanoyl)-D-Lys-Leu-Arg-Pro-Gly-NH_2:

6-D-Lys-GnRH (10 mmol, 12.5 mg) was dissolved in N,N-dimethylformamide (0.5 mL/mg) and DIEA (50 mmol, 9 µL) added. The mixture was stirred briefly (RT) and ß-maleimidopropionic acid N-hydroxysuccinimide ester (MPS; 20 mmol, 5.2 mg) was introduced in one portion. After 30 min reaction time, 10 µL TFA was added to the reaction mixture and the solvent removed in vacuo. The peptide was purified by reverse phase HPLC (Waters PrepPak® 25 x 10™ Delta-Pak™ C_{18}; 10 mL/min; 10-25% B, 0-30 min.; then 25% B, 30-35 min; λ=230 nm).

(N_ε-maleimidopropanoyl)-6-D-Lys-GnRH,FAB-MS (positive ion, NBA matrix) Calc. M+1: 1405.56; Found M+1=1405.6

Conjugation of (N_ε-maleimidopropanoyl)-6-D-Lys-GnRH, to Lys-PE38M, 4:

To a sterile 15 mL polyethylene centrifuge tube with septum was added Lys-PE38M (.221 µmol, 3.0 mL, 2.8 mg/mL) in PBS. The pH of the solution was adjusted to 10.8 by the addition of 350 µL of 1.0 M, pH 11.0 borate buffer. Dithiothreitol (11.0 µmol, 1.7 mg) and ethylenediaminetetraacetic acid disodium salt (22.1 µmol, 8.2 mg) were added and the protein mixture vortexed until all solids were in solution. N-Acetylhomocysteine thiolactone (22.1 µmol, 3.5 mg) was introduced in one portion and the solution degassed and purged with N_2 (degas/purge repeated 5X). The mixture was aged in an N_2 box at RT for 6.5 h, then charged to Spectropor 2 dialysis tubing and dialyzed (RT) as follows: vs. 1) 4L degassed, N_2-sparged, 0.1 M, pH 8.0 phosphate buffer which contained 10 mg EDTA-2 Na and 0.25 mg DTT (16 h); 2) 4L degassed, N_2-sparged, 0.01 M, pH 8.0 phosphate buffer containing 10 mg EDTA-2 Na, 0.25 mg DTT (6h). The thiolated exotoxin was then transferred to a sterile 15 mL polyethylene centrifuge tube (3.90 mL). An Ellman assay on 325 µL of this material indicated that a total of 0.350 µmol of SH was present. To the remaining thiolated material (0.321 mmol SH, 3.57 mL solution) was added 2 (1.60 µmol, 2.25 mg). The reaction was then vortexed briefly and aged in an N_2 box (RT, 1h). The toxin mixture was charged to Spectropor 2 dialysis tubing and dialyzed (4°C) as follows: vs.1) 4 L, 0.01 M, pH 7.0 phosphate buffer (18 h); 2) 4 L, 0.01 M, ph 7.0 phosphate buffer (46 h); and 3) 4 L deionized H_2O (7h). The conjugate was centrifuged to pellet any unsuspended material

and passed through a sterile filter (Millipore 0.22 μm, Millex®-GV). This provided 4, which had HPLC characteristics that were distinct from unconjugated Lys-PE38M.

Lys-PE38M:RP-HPLC (250 mm x 4.6 mm Vydac C_4; 1.5 mL/min.; 36-41% B, 0-30 min.; λ=215 nm): RT = 18.16 min.

EXAMPLE 2

Preparation of PE38M

Plasmid PJH4 (Ref. Hwang. J. Cell (1987, 48; 129-136) contains the coding sequence for PE_{1-613}. Oligonucleotide directed mutagenesis as described in 15.51-15.73, Molecular Cloning, 2nd ed (1989) edited by Sambrook, Fritch & Maniatis (Cold Spring Harbor Press) has been used as a covenient way to make deletions/mutations in the PE molecule. An NDE1/Hind III double digest is carried out on PJH4 resulting in linearization of the construct and clipping of a 12 b_p segment which includes the ATG start codon of the PE coding sequence. Two complementary oligonucleotides are synthesized, annealed and ligated into the NDE1/Hind III splice site. The oligomers have the following nucleotide sequence: 1-5' TAT GCT GCA GGG TAC CAA GCT TAT GGC CGA AGA$^{3'}$ and II - 5' AGC TTC TTC GGC CAT AAG CTT GGT ACC CTG CAG CA3'. The modified PE insert has a sequence of MLQGTKLMAEE constructed at the N-terminus. This plasmid is designated PJH42.

The plasmid PJH42 is partially cut with Ava I. The linear form of DNA is isolated, completely digested with Hind III, and the resulting 5.1 Kb fragment isolated. S1 nuclease treatment is carried out to allow blunt end ligation of the sticky ends and the plasmid is recircularized and designated PJH43. This results in a PE with deletion of amino acids 4-252.

A 553 bp Sal I/Bam H1 fragment of plasmid PJH43 is cloned into M13 mp19. An oligonucleotide, 50 nucleotides in length with the structure 5' GGC GTC GCC GCT GTC CGC CGG GCC GTT GGC CGC GCC GGC CTC GTC GTT GC3', is synthesized and annealed to the single stranded M13 vector to facilitate (loop out) mutagenesis generating a deletion of amino acids 365-380 of the PE insert, resulting in the sequence:

....AGAANGPADSGDALL....
364 381

A 505 b_p Sal I Bam H1 fragment is excised from the replicative form of the mutant DNA in M13 and ligated with a 3.7 Kb Sal 1 Bam H1 fragment of the plasmid PJH43. This new plasmid is designated PJH44.

A Bam H1/EcoR 1 fragment of 460 nucleotides is excised from PJH44 and cloned into M13 mp19. This fragment contains the nucleotide sequence for three lysines that are mutated at the carboxy end of the coding sequence: lysines 590, 606 are mutated to glutamines and lysine 613 is mutated to an arginine.

Oligo directed mutations are then carried out successively at each of the lysines with the following oligomers:

Lysines 590-5' GCT GAT CGC CTG TTC TTG GTC GGG GAT GCT GGA C 3'

Lysines 606-5' GTC CTC GCG CGG CGG TTG GCC GGG CTG GCT G 3'

Lysines 613-5' CGG TCG CGG CAG TTA ACG CAG GTC CTC GCG CGG 3'

The Bam H1 EcoR 1 fragment is excised from the replicative form of the mutant DNA in M13 and ligated with a 3.4 Kb Bam H1/EcoR 1 fragment of the plasmid PJH44. The linearized plasmid is then recircularized, designated PJH45 and used for expression of the modified PE, identified as PE38m, from a commercially available strain of E. coli, HB 101, available from Bethesda Research Laboratories.

EXAMPLE 3

Preparation of other GnRH Analogs

N-Ac-1,2-Di-p-Chloro-Phe-6-DLys-GnRH, Ac-4-Cl-Phe-4-Cl-Phe-Trp-Ser-Tyr-DLys-Leu-Arg-Pro-Gly-NH_2:

The peptide is synthesized on Rink amide MBHA resin (0.25 mmol, Amino Tech) by solid phase peptide synthesis (Fmoc chemistry) using an ABI model 431A synthesizer and double couplings (DCC/HOBT) for 4-Cl-Phe and single couplings for the remaining residues. The amino terminus is capped by treatment

with acetic anhydride (5-10 mL) until the resin beads give a negative Kaiser test for the presence of an amine (0.5-8 h). The peptide is cleaved (2 h-4 h, RT) from the resin using reagent R (0.5 mL-3 mL/100 mg resin, TFA/thioanisole/ethanedithiol/anisole, 90:5:3:2). The peptide is precipitated from the concentrated cleavage mixture with diethyl ether and purified by preparative reverse phase HPLC and characterized by FAB-MS.

6-DLys-10-DAla-GnRH, H-Pgl-His-Trp-Ser-Tyr-DLys-Leu-Arg-Pro-DAla-NH_2:

The peptide was synthesized on Rink amide MBHA resin (0.25 mmol, Amino Tech) by solid phase peptide synthesis (Fmoc chemistry) using an ABI model 431A synthesizer and single couplings (DCC/HOBT). The peptide was cleaved (3 h, RT) from the resin using reagent R (2.0 mL/100 mg resin, TFA/thioanisole/ethanedithiol/anisole, 90:5:3:2). The peptide was precipitated from the concentrated cleavage mixture with diethyl ether and purified by preparative reverse phase HPLC. Characterization by FAB-MS of 6-D-Lys-10-D-Ala-GnRH (positive ion, NBA matrix) Calc (m+1) = 1268.5; Found (m+1) = 1267.5.

6-DLys-9-Pro-NHEt-GnRH, H-Pgl-His-Trp-Ser-Tyr-DLys-Leu-Arg-Pro-NHEt:

The peptide is synthesized on Oxime or Merrifield resin by solid phase peptide synthesis (Boc chemistry) using an ABI model 431A synthesizer and single couplings (DCC/HOBT). The peptide is cleaved (2 h-72 h, RT) from the resin with anhydrous

ethyl amine. The crude protected peptide is precipitated with diethyl ether, collected by suction filtration, and dried overnight (over P_2O_5). The protecting groups are removed from the dry peptide by treatment with anhydrous HF (0°C, 0.5-2 h, 5-30 mL) in the presence of anisole (0.2-2 mL) and dimethyl phosphite (0.1-1 mL). The excess HF is removed in vacuo and the residue triturated with diethyl ether. The peptide is purified by preparative reverse phase HPLC and characterized by FAB-MS.

6-DOrn-GnRH, H-Pgl-His-Trp-Ser-Tyr-DOrn-Leu-Arg-Pro-Gly-NH_2:

The peptide was synthesized on Rink amide MBHA resin (0.25 mmol, Amino Tech) by solid phase peptide synthesis (Fmoc chemistry) using an ABI model 431A synthesizer and single couplings (DCC/HOBT). The peptide was cleaved (3 h, RT) from the resin using reagent R (2.0 mL/100 mg resin, TFA/thio-anisole/ethanedithiol/anisole, 90:5:3:2). The peptide was precipitated from the concentrated cleavage mixture with diethyl ether and purified by preparative reverse phase HPLC. Characterization by FAB-MS of 6-D-Orn-GnRH (positive ion, NBA matrix) Calc (m+1) 1239.4; Found (m+1) 1239.5.

3-Indolylpropionyl-6-DLys-GnRH,3-Indolylpropionyl-Ser-Tyr-DLys-Leu-Arg-Pro-Gly-NH_2:

The peptide was synthesized on Rink amide MBHA resin (0.25 mmol, Amino Tech) by solid phase peptide synthesis (Fmoc chemistry) using an ABI model 431A synthesizer and single couplings (DCC/HOBT).

The peptide was cleaved (3 h, RT) from the resin using reagent R (2.0 mL/100 mg resin, TFA/thioanisole/ethanedithiol/anisole, 90:5:3:2). The peptide was precipitated from the concentrated cleavage mixture with diethyl ether and purified by preparative reverse phase HPLC. Characterization by FAB-MS of 3-indolylpropionyl-6-D-Lys-GnRH (positive ion, NBA matrix) Calc (m+1) 990.2; Found (m+1) 990.7.

3-Indolylpropionyl-6-DLys-9-Pro-NHEt-GnRH, 3-Indolylpropionyl-Ser-Tyr-DLys-Leu-Arg-Pro-NHEt:

The peptide is synthesized on Oxime or Merrifield resin by solid phase peptide synthesis (Boc chemistry) using an ABI model 431A synthesizer and single couplings (DCC/HOBT). The 3-indolylpropionyl moiety is incorporated as N-formyl-3-indolepropionic acid. The peptide is cleaved (2 h-72 h, RT) from the resin with anhydrous ethyl amine. The crude protected peptide is precipitated with diethyl ether, collected by suction filtration, and dried overnight (over P_2O_5). The protecting groups are removed from the dry peptide by treatment with anhydrous HF (0°C, 0.5-2 h, 5-30 mL) in the presence of anisole (0.2-2 mL) and dimethyl phosphite (0.1-1 mL). The excess HF is removed in vacuo and the residue triturated with diethyl ether. The peptide is purified by preparative reverse phase HPLC and characterized by FAB-MS.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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Marburg, Steven
Tolman, Richard L

(ii) TITLE OF INVENTION: Chimeric Toxins Binding to the GnRH Receptor

(iii) NUMBER OF SEQUENCES: 1

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(F) ZIP: 07065

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Rose, David L
(B) REGISTRATION NUMBER: 26,332
(C) REFERENCE/DOCKET NUMBER: 18652

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 355 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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35 40 45

Val Gln Arg Leu Val Ala Leu Tyr Leu Ala Ala Arg Leu Ser Trp Asn
50 55 60

Gln Val Asp Gln Val Ile Arg Asn Ala Leu Ala Ser Pro Gly Ser Gly
65 70 75 80

Gly Asp Leu Gly Glu Ala Ile Arg Glu Gln Pro Glu Gln Ala Arg Leu
85 90 95

Ala Leu Thr Leu Ala Ala Ala Glu Ser Glu Arg Phe Val Arg Gln Gly
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Thr Gly Asn Asp Glu Ala Gly Ala Ala Asn Gly Pro Ala Asp Ser Gly
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Asp Ala Leu Leu Glu Arg Asn Tyr Pro Thr Gly Ala Glu Phe Leu Gly
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                165                 170                 175

Val Phe Val Gly Tyr His Gly Thr Phe Leu Glu Ala Ala Gln Ser Ile
            180                 185                 190

Val Phe Gly Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala Ile Trp
        195                 200                 205

Arg Gly Phe Tyr Ile Ala Gly Asp Pro Ala Leu Ala Tyr Gly Tyr Ala
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Gln Asp Gln Glu Pro Asp Ala Arg Gly Arg Ile Arg Asn Gly Ala Leu
225                 230                 235                 240

Leu Arg Val Tyr Val Pro Arg Ser Ser Leu Pro Gly Phe Tyr Arg Thr
                245                 250                 255

Ser Leu Thr Leu Ala Ala Pro Glu Ala Ala Gly Glu Val Glu Arg Leu
            260                 265                 270

Ile Gly His Pro Leu Pro Leu Arg Leu Asp Ala Ile Thr Gly Pro Glu
        275                 280                 285

Glu Glu Gly Gly Arg Leu Glu Thr Ile Leu Gly Trp Pro Leu Ala Glu
    290                 295                 300

Arg Thr Val Val Ile Pro Ser Ala Ile Pro Thr Asp Pro Arg Asn Val
305                 310                 315                 320

Gly Gly Asp Leu Asp Pro Ser Ser Ile Pro Asp Gln Glu Gln Ala Ile
                325                 330                 335

Ser Ala Leu Pro Asp Tyr Ala Ser Gln Pro Gly Gln Pro Pro Arg Glu
            340                 345                 350

Asp Leu Arg
        355
```

WHAT IS CLAIMED IS:

1. A toxin conjugate having the formula:

$$Q\text{-Ser-Tyr-W-X-Arg-Y-Z}$$
$$\begin{matrix} L_1 \\ L_2 \\ T \end{matrix}$$

where

Q is PyroGlu-His-Trp, N-acetyl-4-Cl-Phe^{1,2}-Trp, or 3-indolylpropionyl;

W is the D or L amino acid

$$-C(=O)-CH(NH)-(CH_2)_r-B-(CH_2)_m-N(R_1)-(\text{amino acid})_n-;$$

where

r is 1 or 2;

m is 1 to 4;

n is 0 or 1;

B is CH_2, O, S or N; and

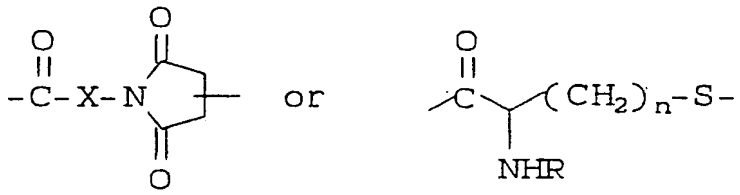
R_1 is hydrogen, C_1-C_6alkyl, or C_3-C_8cycloalkyl;

X is Leu or Nle;

Z is Gly-NH_2, ethylamide or Aza-Gly-NH_2;

Y is Pro or 4-hydroxy Pro;

L_1 and L_2 are independently



where X is C_1-C_5 alkylene, phenyl, or C_5-C_6 cycloalkylene;

R is C_1-C_3 alkanoyl; and

n is 1 or 2; and

T is a toxin group; provided that the carboxyl end of each of L_1 and L_2 are bonded to either the GnRH derivative or to the toxin.

2. A toxin conjugate of Claim 1 having the formula:

PyroGlu-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Y-Z

L_1

L_2

T

where Y, Z, L_1, L_2 and T are as defined Claim 1.

3. The toxin conjugate of Claim 2 where the toxin is capable of destroying the LH releasing cells of the pituitary gland.

4. The toxin conjugate of Claim 3 where the toxin is a plant derived toxin, a bacteria derived toxin or a chemical toxin.

5. The toxin conjugate of Claim 4 where the plant derived toxin is ricin, modeccin, abrin, pokeweed antiviral protein, α-amantin, gelonin ribosome inhibiting protein (RIP) or RIP derived from wheat, corn, rye or flax.

6. The toxin conjugate of Claim 4 where the chemical toxin is melphalan methotrexate, nitrogen mustard, doxorubicin or daunomycin.

7. The toxin conjugate of Claim 4 where the bacteria derived toxin is diphtheria toxin, Pseudomonas exotoxin or shiga toxin.

8. The toxin conjugate of Claim 7 where the bacteria derived toxin is Pseudomonas exotoxin or a segment thereof.

9. The toxin conjugate of Claim 8 where the Pseudomonas exotoxin has been modified such that the binding domain has been partly or completely deleted.

10. The toxin conjugate of Claim 9 where the Pseudomonas exotoxin has been modified to delete amino acids 1-252 and retain amino acid 253-613.

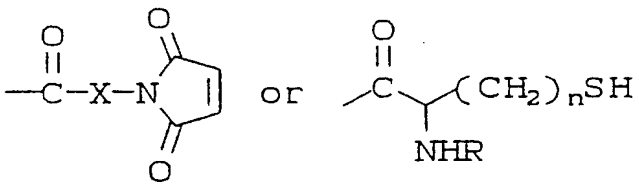
11. A process for the preparation of a compound of Claim 1 which comprises treating a GnRH derivative having the formula:

Q-Ser-Tyr-W-X-Arg-Y-Z
L_1'

where Q, W, X, Y and Z are defined in Claim 1 with toxin having the formula

L_2' - T

where T is a toxin and L_1' and L_2' independently are



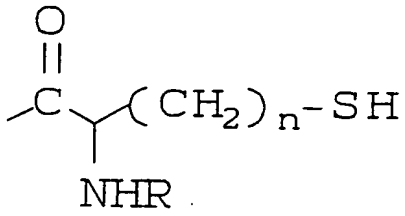
where X, R and n are as defined in Claim 1.

12. The process of Claim 11 where the GnRH derivative has the formula:

PyroGlu-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Y-Z
L_1'

13. The process of Claim 12 where the GnRH derivative is bonded to L_1' having the formula

and the toxin T is bonded to L_2' having the formula



14. The process of Claim 13 where the GnRH derivative is D-Lys^6-GnRH.

15. The process of Claim 13 where the toxin T is a Pseudomonas exotoxin.

16. The process of Claim 14 where the Pseudomonas exotoxin is PE-38M.

17. A method for the sterilization of animals which comprises administering to such animals an effective amount of a toxin conjugate of Claim 1.

18. A composition useful for the sterilization of animals which comprises an inert carrier and an effective amount of a toxin conjugate of Claim 1.

19. A GnRH derivative having the formula

Q-Ser-Tyr-W-X-Arg-Y-Z

where

Q is PyroGlu-His-Trp-, N-acetyl-4-Cl-Phe^{1,2}-Trp, or 3-indolylpropionyl;

W is the D or L amino acid

$$-C(=O)-N-(CH_2)_r-B-(CH_2)_m-NHR_1;$$

where

r is 1 or 2;

m is 1 to 4;

B is CH_2, O, S or N; and

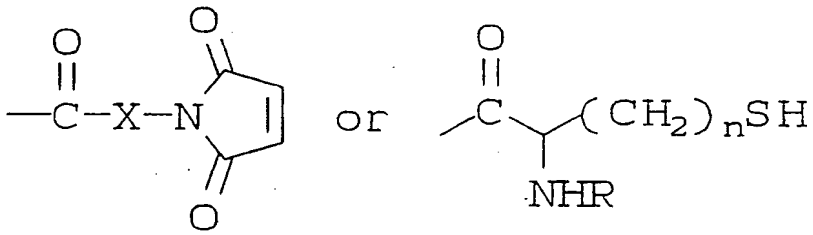
R_1 is hydrogen, C_1-C_6alkyl, C_1-C_6alkanoyl, C_3-C_8cycloalkyl or an amino acid linked through its C-terminus;

X is Leu or Nle;

Y is Pro or 4-hydroxyPro;

Z is Gly-NH_2, ethylamide or Aza-Gly-NH_2; and

L is



where

X is C_1-C_5-alkylene, phenyl or C_5-C_6 cycloalkylene;

R is C_1-C_3-alkanoyl; and

n is 1 or 2.

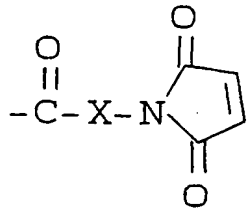
20. A GnRH derivative of Claim 19 having the formula

PyroGlu-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Y-Z

L

where Y, Z and L are as defined in Claim 19.

21. A compound of Claim 19 where Y is Pro and L is

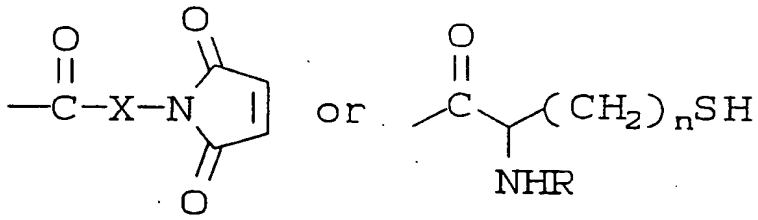


and X is as defined in Claim 19.

22. A compound having the formula

L-T

where T is a toxin and L is



where

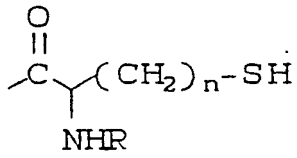
X is C_1-C_5 alkylene, phenyl, or C_5-C_6 cycloalkylene;

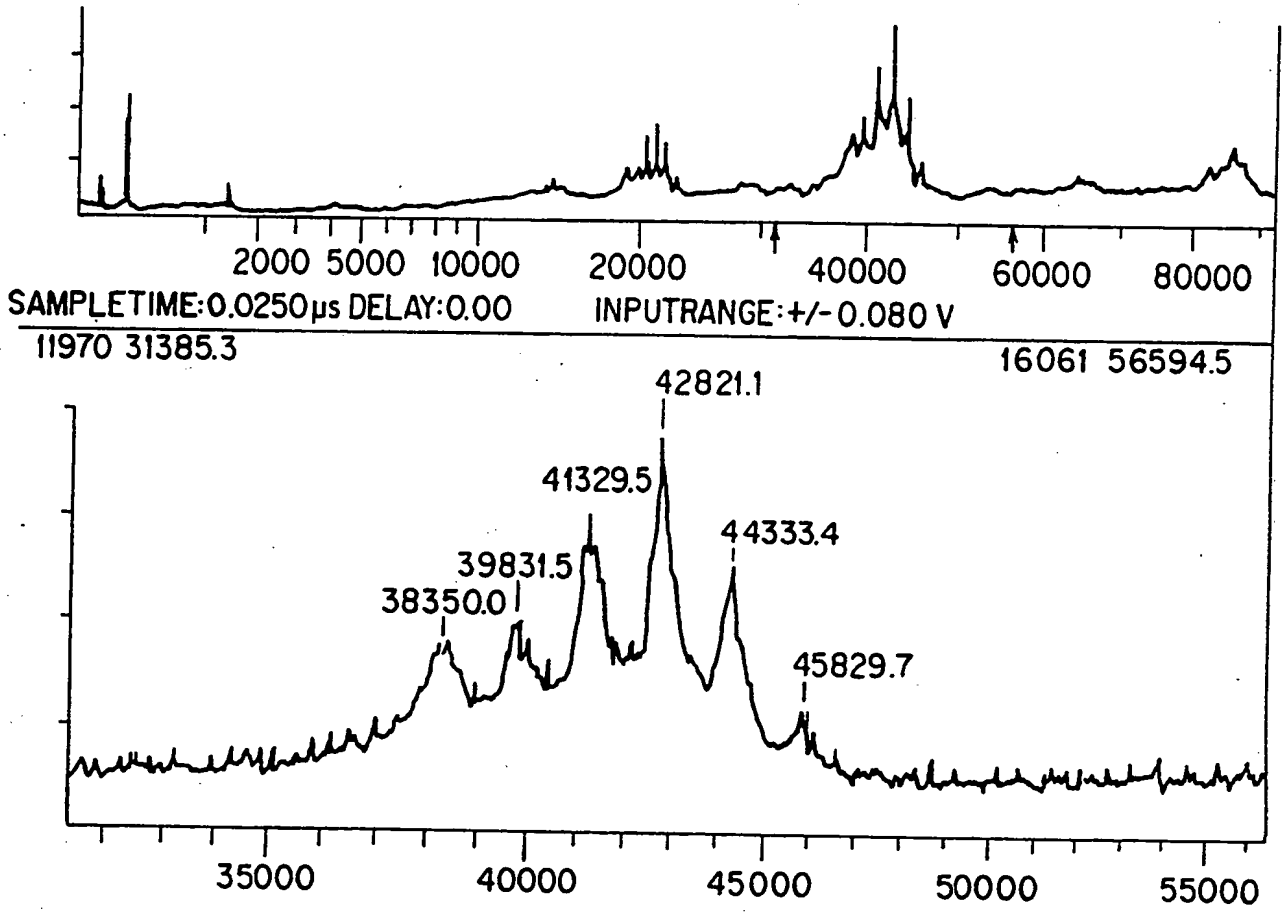
R is C_1-C_3 alkanoyl; and

n is 1 or 2; and

23. A compound of Claim 22 where T is a Pseudomonas exotoxin.

24. A compound of Claim 23 where T is PE38M and L is





MASS SPECTROMETRY
LASER-TOF TECHNIQUE
MATRIX ASSISTED MATRIX DESORPTION TOF MS
MS OF CONJUGATE NewLys 38+ D-LYS^6 GnRH

four Lys's
1 to 5 GnRH's / PROTEIN

FIG. 1

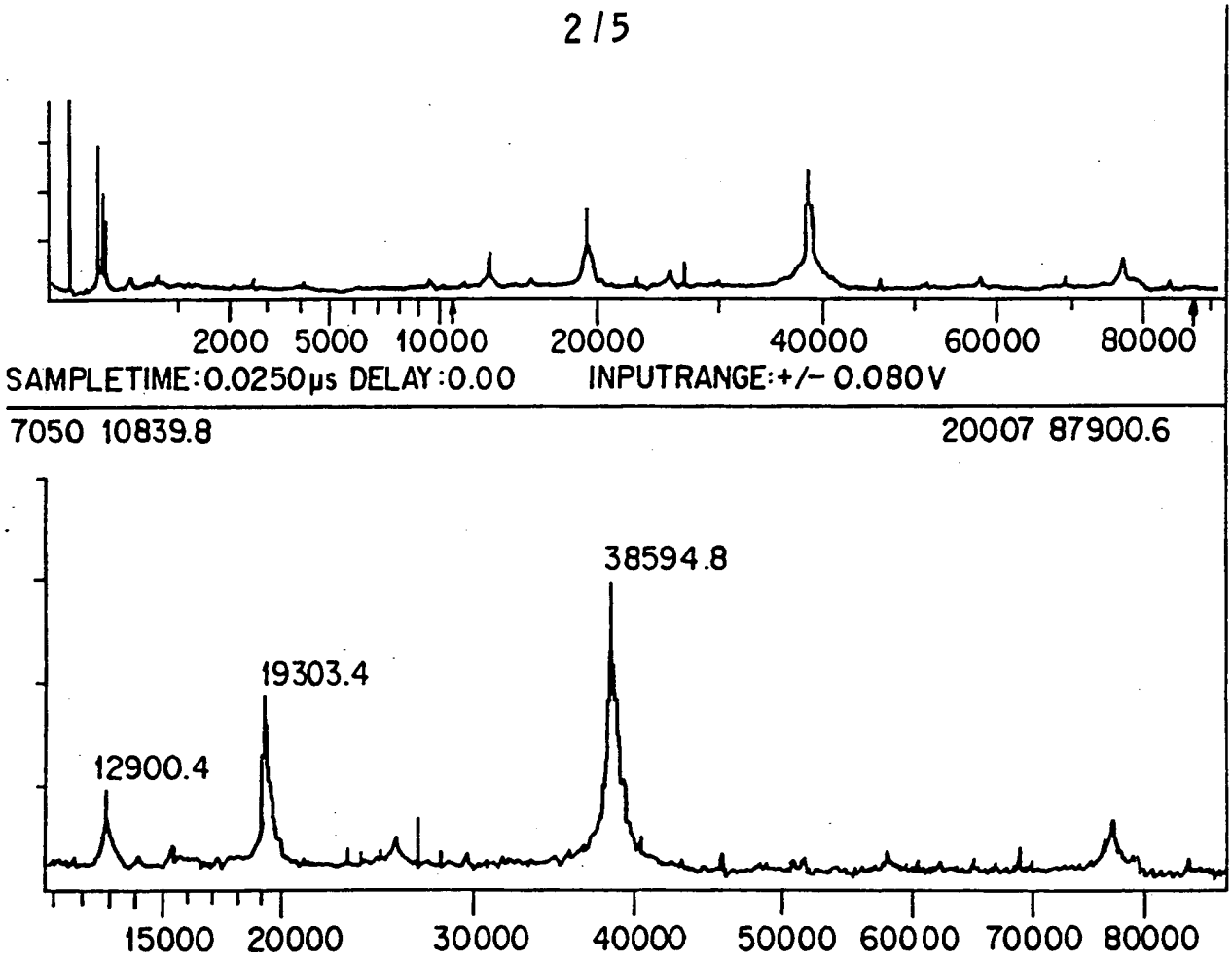

2000 5000 10000 20000 40000 60000 80000
SAMPLETIME:0.0250µs DELAY:0.00 INPUTRANGE:+/- 0.080V
7050 10839.8
20007 87900.6
38594.8
19303.4
12900.4
15000 20000 30000 40000 50000 60000 70000 80000
STARTING MATERIAL (TOXIN ONLY)
NewLys38
19303 DOUBLY CHARGED
12900 TRIPLY CHARGED

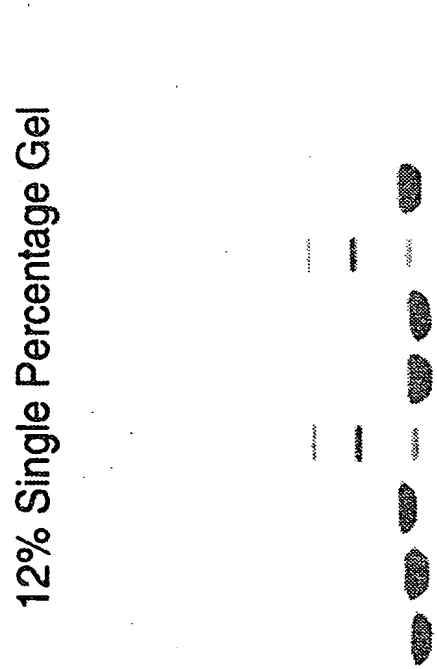
FIG. 2

Lane	Identitiy
1,10	Blank
2,6,8	M.W. Standards
3	Conjugate LysPE38 and 6-D-Lys-GnRH, 1.8µg
4	Conjugate LysPE38 and 6-D-Lys-GnRH, 2.3µg
5	Conjugate LysPE38 and 6-D-Lys-GnRH, 3.5µg
7	Lys PE38, 1.8 µg
9	Conjugate LysPE38M and 6-D-Lys-GnRH, 5µg

FIG. 3

10% Single Percentage Gel
1 2 3 4 5 6 7 8 9 10
Lane
Identitiy
1 Blank
2,4,7,10 M.W. Standards
3 Conjugate LysPE38 and 6-D-Lys-GnRH, 1.4µg
5 LysPE38, 2.6 µg
6 Conjugate LysPE38 and 6-D-Lys-GnRH, 2.3µg
8 Lys PE38, 4.0 µg
9 Conjugate LysPE38 and 6-D-Lys-GnRH, 3.5µg

FIG. 4



Lane	Identitiy
1,10,11,12	Blank
2	LysPE38M, 3µg
3	Conjugate LysPE38M and 6-D-Lys-GnRH, 3µg
4	TP40, 3µg
5	MW stds.
6	Conjugate LysPE38M and 6-D-Lys-GnRH, 3µg
7	Lys PE38M, 3 µg
8	MW stds.
9	Conjugate LysPE38M and 6-D-Lys-GnRH, 3µg

FIG. 5

INTERNATIONAL SEARCH REPORT

National application No.
PCT/US93/01263

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/00, 37/02; CO7K 5/00, 7/00, 15/00, 17/00
US CL :530/328; 514/15
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/328; 514/15

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, CA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 90/09799 (NETT ET AL.) 07 SEPTEMBER 1990, SEE ENTIRE DOCUMENT.	1-24
Y	US, A, 4,855,285 (STEVENS) 08 AUGUST 1989, SEE ENTIRE DOCUMENT.	1-24
Y	GB, A, 2,228,262 (TALWAR ET AL.) 22 AUGUST 1990, SEE ENTIRE DOCUMENT.	1-24

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
27 April 1993

Date of mailing of the international search report
04 MAY 1993

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